

SEMIOCHEMICALS OF THE SCARABAEINAE. VII: IDENTIFICATION AND SYNTHESIS OF EAD-ACTIVE CONSTITUENTS OF ABDOMINAL SEX ATTRACTING SECRETION OF THE MALE DUNG BEETLE, *Kheper subaeneus*

B. V. BURGER, W. G. B. PETERSEN, W. G. WEBER, and Z. M. MUNRO

Laboratory for Ecological Chemistry Department of Chemistry University of Stellenbosch Stellenbosch 7600, South Africa

Abstract: Using gas chromatography with flame ionization detection (FID) and electroantennographic detection (EAD) in parallel, butanoic acid, skatole, and (*E*)-2,6-dimethyl-6-octen-2-ol were identified as constituents of the abdominal sex-attracting secretion of the male dung beetle, *Kheper subaeneus*, which reproducibly elicited EAD responses in male and female antennae. This is the first report of the occurrence of (*E*)-2,6-dimethyl-6-octen-2-ol as a natural product, for which the name (*E*)-subaeneol is proposed. In some experiments, a few other constituents of the secretion also gave reproducible responses in specific male and female antennae but did not elicit responses when the analyses were repeated with other antennae. The major volatile constituent of the secretion, identified as (*S*)-(C)-2,6-dimethyl-5-heptenoic acid, is one of these EAD-active compounds. Both this compound and (*E*)-2,6-dimethyl-6-octen-2-ol were synthesized from authentic starting materials for comparison with the natural products.

Key Words—Scarabaeinae, *Kheper subaeneus*, dung beetle, sex attractant, insect semiochemicals, electroantennography, terpenes, semiochemical synthesis.

INTRODUCTION

Male ball-rolling dung beetles of the genus *Kheper* produce a white, flocculant proteinaceous carrier material containing minute amounts of complex mixtures of volatile organic compounds acting as sex attractants for females. The secretion is extruded from several hundred tiny openings in a depression on either side of the first abdominal sternite. It is then broken up into fine particles and released into the air by brushes on the tibia of the male. The ecological importance of dung beetles in general and biological aspects of the production and function of the abdominal sex attractant secreted by male *Kheper* dung beetles has been discussed in previous communications on this subject (Burger et al., 1983, 1990, 1995; Burger and Petersen, 1991, 2002).

Hexadecanoic acid, 2,6-dimethyl-5-heptenoic acid, (*E*)-nerolidol, and skatole, were previously identified as the major volatile organic constituents of the abdominal secretion of *K. lamarcki*. (Burger et al., 1983). The abdominal secretion of *K. nigroaeneus* contains the EAD-active constituents (*R*)-3-methylheptanoic acid and skatole. A third EAD-active constituent of this secretion was not identified, as it is present in such a low concentration that it could barely be detected by gas chromatography (GC) using flame ionization detection (FID) (Burger and Petersen, 2002). A third *Kheper* species, *K. subaeneus*, occurs in parts of the Hluhluwe Game Reserve, about 50 km from the Mkuzi Game Reserve (both in South Africa) where *K. nigroaeneus* and *K. lamarcki* are found. In this communication, we report the identification of the EAD-active constituents of the abdominal sex-attracting secretion of male *K. subaeneus*.

METHODS AND MATERIALS

Collection of Material

We initially attempted to catch *K. subaeneus* dung beetles in pitfall traps baited with rhinoceros or horse dung in Hluhluwe Game Reserve, a method that was used effectively to catch *K. nigroaeneus* in Mkuzi Game Reserve. Probably due to the availability of large quantities of rhinoceros dung in the parts of the game reserve where these beetles are found, this method proved ineffective in catching *K. subaeneus* in Hluhluwe. *K. subaeneus* were, therefore, collected annually during the first week of November from 1988 to 1995 from rhinoceros dung middens along dirt roads near the main entrance gate of Hluhluwe Game Reserve. The dung beetles were transported to Stellenbosch, South Africa, and were kept in greenhouses. The secretion was collected as described in the previous paper in this series on semiochemicals of the Scarabaeinae (Burger and Petersen, 2002).

Analytical Methods

Gas chromatographic determinations were carried out with a Carlo Erba 4160 gas chromatograph with parallel flame ionization and electroantennographic detection (FID/EAD). The instrument was equipped with a 40-m x 0.3-mm glass capillary column coated with OV-1701-OH at a film thickness of 0.375 µm. Helium was used as carrier gas at a linear velocity of 28.5 cm/sec at 40°C, and a temperature program of 2°C/min from 40°C to 250°C (hold) was employed. Chiral gas chromatographic analyses were done on a glass column (25 m x 0.3 mm) coated with 10% heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin in OV-1701-OH at a film thickness of 0.25 µm, using H₂ as carrier gas at a linear velocity of 50 cm/sec. The column was programmed at 2°C/min from 40°C to 190°C (hold). The instrumentation for GC analysis of dung

beetle secretions with FID and EAD recording in parallel using dung beetle antennae as sensing elements has been described by Burger and Petersen (1991). In short, an antenna was inserted lengthwise into the air duct, as this arrangement produced a less noisy baseline than positioning the antenna sideways in the airstream, a position in which the club of the antenna is subjected to the effect of the turbulent mixing of the effluent and ambient air.

The antenna was protected against heat radiation from the gas chromatograph by a heat shield cooled with water at 18°C. Electrical responses in the antenna were recorded via Ag–AgCl electrodes placed in pipets filled with saline solution containing NaCl (7.5 g/liter), CaCl₂ (0.21 g/liter), KCl (0.35 g/liter), and NaHCO₃ (0.2 g/liter), as well as polyvinylpyrrolidone K90 (Fluka, 49 g/liter) to increase its viscosity (Van der Pers, 1980). Antennal responses were amplified with a Murphy Developments AMS-025 amplifier and were recorded on an Omniscribe recorder. Over a period of about 5 years, EAD/FID analyses were done with at least 80 male and as many female insects. Only one antenna of each dung beetle was used so that the beetle could still be used for the collection of secretion. Mass spectra were recorded on a Carlo Erba QMD 1000 quadrupole mass spectrometer using the column and gas chromatographic conditions specified above. ¹H and ¹³C NMR spectra of synthetic compounds and intermediates were recorded at 299.905 and 75.42 MHz, respectively, at 25°C on a Varian VXR 300 NMR spectrometer.

Reference Compounds.

Commercially available reference compounds and starting compounds for the synthesis of other reference compounds were purchased from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), or Sigma-Aldrich (Cape Town, South Africa). (*E*)-2,6-Dimethyl-6-octen-2-ol (**4a**) was prepared from geraniol (3,7-dimethyl-2,6-octadien-1-ol) (**1a**) according to Scheme 1. Tetrachloromethane (69.3 g; 450 mmol) was added during 90 min to a stirred and chilled (0°C) solution of geraniol (**1a**) (61.6 g; 400 mmol) and triphenylphosphine (115.3 g; 440 mmol) in dry acetonitrile (350 ml). The temperature of the reaction mixture was allowed to rise gradually to 22°C at which temperature it was stirred for a further 19 hr. Pentane (300 ml) was added to the reaction mixture, which was stirred at room temperature for 15 min and left overnight at –15°C to allow precipitation of triphenylphosphine oxide.

The precipitate was removed by filtration of the cold suspension. Pentane and acetonitrile were removed on a rotary evaporator, the remaining material diluted with pentane, the solution cooled to –15°C, and the precipitate filtered off. This procedure was repeated until no further triphenylphosphine oxide precipitated. Removal of the solvent and repeated fractionation gave a colorless oil (27.4 g; 39.7%), bp 106–107°C/22 mm Hg, containing (*E*)-8-chloro-2,6-dimethyl-2,6-octadiene (**2a**) in 97.6% purity (GC-MS). MS: *m/z* (%) = 159(0.3), 157(1), 136(10), 129(4), 121(9), 105(5), 93(57), 91(26), 79(25), 77(23), 69(100), 68(23), 67(23), 53(14), 41(53), 39(30). ¹³C NMR (CDCl₃): δ = 41.17 (t, C-1); 120.31 (d, C-2); 142.78 (s, C-3); 39.47 (t, C-4); 26.25 (t, C-5); 123.61 (d, C-6); 131.99 (s, C-7); 25.68 (q, C-8); 16.12 (q, CH₃C-3); 17.71 (q, CH₃C-7).

(*E*)-8-Chloro-2,6-dimethyl-2,6-octadiene (**2a**) (27.4 g; 159 mmol) was slowly added to a stirred solution of lithium aluminum hydride in tetrahydrofuran (THF) (380 ml; 0.02 g/ml; 200 mmol). The reaction mixture was refluxed for 4 hr, allowed to cool to room temperature, and diluted with pentane (150 ml). The precipitated inorganic material was filtered off. Conventional work-up procedures and distillation of the product gave a colorless oil (16.9 g; 77.2%); bp 77°C/40 mm Hg, containing 98.0% (GC-MS) of (*E*)-2,6-dimethyl-2,6-octadiene (**3a**); MS: *m/z* (%) = 138(3), 123(15), 109(1), 107(2), 95(38), 81(3), 79(3), 77(2), 69(100), 67(11), 55(5), 53(7), 41(53), 39(8). ¹³C NMR (CDCl₃): δ = 26.38 (q, C-1); 131.98 (s, C-2); 125.11 (d, C-3); 27.45 (t, C-4); 40.42 (t, C-5); 136.49 (s, C-6); 118.93 (d, C-7); 14.02 (q, C-8); 18.34 (q, CH₃C-2); 16.34 (q, CH₃C-6). Solid mercury(II) acetate (3.2 g; 10 mmol) was added in one batch to a stirred solution of the diene (**3a**) (1.38 g; 10 mmol) in THF (40 ml) and water (10 ml) at 22°C to produce a yellow reaction mixture (Brown et al., 1972).

The reaction mixture became colorless after 6 min, and it was stirred for a further 2 hr. A solution of sodium hydroxide (1.2 g NaOH in 10 ml H₂O) was added in one batch to the reaction mixture, followed immediately by the rapid addition of a solution of sodium borohydride and sodium hydroxide (0.19 g NaBH₄ and 1.2 g NaOH in 10 ml H₂O). The temperature of the reaction mixture rose to 38°C, and mercury started to precipitate. The reaction mixture was stirred for 30 min, after which the supernatant solution was decanted from the mercury and saturated with K₂CO₃, resulting in the separation of the THF layer containing the organic material from the saturated K₂CO₃ solution. Careful concentration of the THF phase on a rotary evaporator gave a mixture of the organic products (1.41 g) containing the starting material (**3a**), an unidentified product that could possibly be a cyclic ether formed from the starting material (Brown et al., 1972), the two terpenols (**4a**) and (**5**), and 2,6-dimethylocta-2,6-diol (**6**) in a ratio of 24:33:29:3:11.

This procedure was repeated several times and the products (10.4 g) combined for column chromatographic fractionation on silica gel (450 g, Merck 60, 230–400 mesh). Elution with 10% ether in hexane gave a fraction (1.16 g) containing the desired terpenol (**4a**) and the terpenol (**5**) in a ratio of 97:3 (GC-MS). This fraction was subjected to bulb-to-bulb distillation (135°C/25 mm Hg) for spectrometric characterization. The major constituent was 2,6-dimethyl-6-octen-2-ol (**4a**); *m/z* (%) = (138)7, 123(20), 109(3), 95(6), 83(19), 82(100), 69(23), 67(80), 59(57), 55(23), 43(30), 41(3). ¹³C NMR (CDCl₃): δ = 29.87 (q, C-1); 71.67 (s, C-2); 44.17 (t, C-3); 23.31 (t, C-4); 40.67 (t, C-5); 136.31 (s, C-6); 119.15 (d, C-7); 13.95 (q, C-8); 29.87 (q, CH₃C-2); 16.14 (q, CH₃C-6). The minor constituent was 3,7-dimethyl-6-octen-3-ol (**5**); *m/z* (%) = 156(2),

141(17), 138(9), 127(8), 123(40), 113(13), 109(18), 95(13), 85(19), 81(18), 71(100), 69(25), 67(23), 58(19), 55(30), 53(12), 43(68), 41(45), 39(20).

(*R*)-(-)-2,6-Dimethyl-5-heptenoic acid (**10a**), was prepared from (*R*)-(-)-3,7-dimethyl-1,6-octadiene [(*-*)- β -citronellene] (**7a**) according to Scheme 2. A solution of 3-chloroperbenzoic acid (containing 6% 3-chlorobenzoic acid) (8.23 g; 44.85 mmol) in chloroform (100 ml) was added over 1.25 hr to (*R*)-(-)-3,7-dimethyl-1,6-octadiene (**7a**) in chloroform (40 ml) at such a rate that the temperature of the reaction mixture did not exceed -10°C . The progress of the partial epoxidation of the β -citronellene was monitored by GC analysis and was completed after stirring the reaction mixture an additional 2 hr at -10°C . The 3-chlorobenzoic acid was filtered off, and the temperature of the filtrate allowed to rise to room temperature. The filtrate was washed with NaOH solution (0.45 M, 40 ml), Na_2CO_3 solution (5%), saturated brine and dried. Removal of the solvent under reduced pressure gave a colorless oil (6.87 g; 99.3%) containing 95.9% (GC-MS) of (*R*)-(-)-6,7-epoxy-3,7-dimethyl-1-octene (**8a**). MS: m/z (%) = 139(4), 125(2), 121(3), 111(6), 95(14), 85(13), 82(16), 81(100), 79(18), 71(30), 69(39), 68(50), 67(60), 59(64), 55(53), 54(40), 53(20), 43(44), 41(64), 39(37).

Ozone was passed through a solution of the epoxide (**8a**) (6.22 g, 40.32 mmol) in dry dichloromethane (350 ml) at -70°C until, after about 35 min, the reaction mixture developed a light purple color. The excess of ozone and oxygen was removed as quickly as possible by passing nitrogen through the reaction mixture for 40 min. This solution was carefully concentrated to 40 ml, cooled to 0°C , and added to a cold suspension of zinc (15.0 g; 229.4 mmol) in acetic acid (39 ml) containing sodium acetate (3.41 g; 41.52 mmol) and sodium iodide (10.72 g; 68.64 mmol) (Knöll and Tamm, 1975). The bulk of the dichloromethane was removed under reduced pressure, and the resulting solution was stirred for 2 hr at room temperature. The zinc was filtered off, and the solution of the reaction products in acetic acid was extracted repeatedly with dichloromethane. The combined extracts were washed with saturated brine and water, dried, and the solvent removed to yield a liquid product (5.52 g; 97.7%) containing 71.5% (GC-MS) of the target product, (*R*)-(-)-2,6-dimethyl-5-heptenal (**9a**). MS: m/z (%) = 140(3), 107(2), 97(1), 93(1), 91(2), 83(12), 82(100), 81(7), 70(5), 69(24), 67(46), 57(5), 56(16), 55(21), 53(10), 43(8), 42(7), 41(51), 39(16).

(*R*)-(-)-2,6-Dimethyl-5-heptenal (**9a**) was oxidized to the corresponding acid with chromic acid. Chromic acid solution (24 ml, containing 4.79 g of CrO_3 in 3 M H_2SO_4) was slowly added to a stirred solution of (*R*)-(-)-2,6-dimethyl-5-heptenal (**9a**) (3.85 g; 27.46 mmol) in acetone (40 ml). The color of the reaction mixture changed from orange to a dirty green, and a precipitate of inorganic salts was formed. The acetone solution of the organic products was decanted from the inorganic material, the acetone evaporated under reduced pressure, and the residual material dissolved in dichloromethane. The inorganic precipitate was dissolved in water (100 ml) and repeatedly extracted with dichloromethane. All of the dichloromethane extracts were combined and extracted with NaOH solution (3 M; 30 ml). This basic solution was extracted five times with dichloromethane to remove all of the unsaponified material, after which it was acidified with dilute sulfuric acid (3 M; 5.5 ml) and the organic acid extracted with dichloromethane.

Conventional work-up methods and distillation gave the target compound, (*R*)-(-)-2,6-dimethyl-5-heptenoic acid (**10a**) (2.07 g) in a yield of 48.3%, bp $112\text{--}114/5$ mm Hg, $[\alpha]_{25}^{\text{D}} = -18.27^{\circ}$ (neat), 95.7% ee (GC). MS: m/z (%) = 156(9), 138(13), 110(4), 95(7), 83(88), 82(49), 74(58), 69(46), 67(38), 55(48), 53(12), 41(100), 39(30). ^{13}C NMR (CDCl_3): δ = 183.04 (s, C-1); 38.84 (d, C-2); 33.56 (t, C-3); 25.62 (t, C-4); 123.48 (d, C-5); 132.42 (s, C-6); 25.68 (q, C-7); 16.86 (q, $\text{CH}_3\text{C-2}$); 17.65 (q, $\text{CH}_3\text{C-6}$). (*S*)-(+)-2,6-Dimethyl-5-heptenoic acid (**10b**) was prepared from (+)- β -citronellene (**7b**) according to Scheme 2 and the procedures described for its *R* enantiomer. (*S*)-(+)-6,7-Epoxy-3,7-dimethyl-1-octene (**8b**) was obtained as a colorless oil (6.73 g; 97.3%) in a purity of 96.8% and converted to (*S*)-(C)-2,6-dimethyl-5-heptenal (**9b**), which was used without purification for the preparation of (*S*)-(+)-2,6-dimethyl-5-heptenoic acid (**10b**) (2.12 g; 48.1%). bp $117\text{--}118/5$ mm Hg; $[\alpha]_{25}^{\text{D}} = +24.6^{\circ}$ (neat), 96.3% ee (GC). MS: m/z (%) = 156(10), 138(14), 110(4), 95(7), 83(97), 82(52), 74(65), 69(48), 67(43), 55(50), 53(11), 41(100), 39(28). ^{13}C NMR (CDCl_3): δ = 183.02 (s, C-1); 38.82 (d, C-2); 33.55 (t, C-3); 25.61 (t, C-4); 123.45 (d, C-5); 132.44 (s, C-6); 25.68 (q, C-7); 16.86 (q, $\text{CH}_3\text{C-2}$); 17.65 (q, $\text{CH}_3\text{C-6}$).

RESULTS AND DISCUSSION

In their natural habitat, male *K. subaeneus* can often be observed exhibiting the typical secretion-producing behavior, which can be summarized as follows. Oriented with the head upwind and the front two pairs of legs on the dung surface, the male raises his hind legs and lowers his head into the dung. The legs are retracted simultaneously inwards towards the sides of the body and are then simultaneously extended. This movement results in the tibial brushes brushing the controlled-release proteinaceous material (Burger et al., 1990), extruded from the abdomen as fine fibers and containing the sex attractant, against the rows of curved abdominal bristles. This causes puffs of attractant to rise into the air from both sides of the insect. After a short time of approximately 20–30 sec, the retraction and extension of the hind legs are repeated. This procedure is kept up until a female arrives or the male is disturbed by other insects in or on the dung (Burger et al., 1983).

Although their population density was rather low in Hluhluwe Game Reserve during the late 1980s, five or more males were often found calling on one large rhinoceros dung midden. In contrast, calling behavior has not been observed with the same frequency in *K. lamarcki* in Mkuzi Game Reserve. In captivity, however, this situation was reversed, and the secretion of male *K. subaeneus* was collected only in small quantities on only a few occasions. A total of only a few milligrams were collected over a period of 5 years from about 700 males. A gas chromatogram of the volatile organic fraction of the abdominal secretion of *K. subaeneus* is shown in Figure 1. The general appearance of this gas chromatogram is similar to that of *K. nigraeneus* (Burger and Petersen, 2002) in that both secretions contain mostly heavy constituents. However, only a relatively small percentage of the compounds present are common to both secretions. A typical GC analysis with FID and EAD recording in parallel using a female antenna as sensing element is shown in Figure 2.

The constituent eluting at a retention time of 4.98 min was identified as butanoic acid (*n*-butyric acid) from its mass spectrum and coelution with the synthetic compound. Although the EAD response is weak, it was present in all EAD chromatograms. The constituent eluting at 28.27 min was identified as 3-methylindole (skatole) and is also present in the abdominal secretions of *K. lamarcki* (Burger et al., 1983) and *K. nigraeneus* (Burger and Petersen, 2002). Although barely detectable by FID, the constituent eluting at 17.75 min gave a strong and reproducible EAD response. This constituent elutes with a shorter retention time than the EAD-active constituent that remained unidentified in the male abdominal secretion of *Kheper nigraeneus* (Burger and Petersen, 2002). Male antennae produced similar EAD/FID analyses.

After the gas chromatographic analyses had been completed, only about ten times the quantity used for the analysis shown in Figure 2 was available for further analytical work. All of this material, collected during the previous five summer seasons, was used in a final attempt at obtaining some mass spectral information on the structure of the constituent eluting at 17.75 min. Using a very high electron multiplier setting, the mass spectrum shown in Figure 3 was obtained. The spectrum contains several ions typically found in the spectra of the monoterpenes. This and the presence of compounds with terpenoid character in the abdominal secretions of other *Kheper* species prompted us to compare the retention time and mass spectrum of the unknown compound with those of selected model terpenoid compounds.

It became clear that the unknown compound was not a cyclic or acyclic monoterpene or a primary monoterpene alcohol and that it could probably be a tertiary monoterpene alcohol, albeit not one of the tertiary terpenoid alcohols that we had available for comparison. Partial hydroxylation of (*E*)- and (*Z*)-3,7-dimethyl-1,6-octadiene with mercury(II) acetate gave a mixture of alcohols containing, among others, 2,6-dimethyl-7-octen-2-ol, which had a retention time similar to that of the EAD-active constituent of the secretion but a slightly different mass spectrum. It was, therefore, concluded that the unknown constituent could possibly be a similar tertiary alcohol but with its double bond in a different position. Conversion of a mixture of geraniol (**1a**) and nerol (**1b**) [(*E*)- and (*Z*)-3,7-dimethyl-2,6-octadien-1-ol, respectively], via the corresponding chlorides (**2a**) and (**2b**), to (*E*)- and (*Z*)-2,6-dimethyl-2,6-octadiene, (**3a**) and (**3b**), respectively (Scheme 1), and subjecting this mixture to partial hydroxylation, gave a mixture containing the two unchanged terpenes (**3a** and **3b**), the two terpenols (**4a** and **4b**), the terpenol (**5**), and the terpenediol (**6**).

One of the terpenols was EAD-active and had the retention time and mass spectrum of the unknown compound. From the relative retention times of the three terpenols, it was concluded that the EAD-active one could possibly be (*E*)-2,6-dimethyl-6-octen-2-ol (**4a**). The configuration of the double bond in the EAD-active compound was confirmed by repeating the synthesis with geraniol according to Scheme 1. Geraniol [(*E*)-3,7-dimethyl-2,6-dien-1-ol] (**1a**) was, thus, converted to (*E*)-8-chloro-2,6-dimethyl-2,6-octadiene (**2a**). Reduction of this chloride with lithium aluminium hydride gave the diene (**3a**). Partial hydroxylation of the diene (**3a**) by mercuriation-demercuration according to Brown and Geoghegan, (1967) i.e., by the addition of the diene to a quantity of mercury(II) acetate equivalent to the hydroxylation of one of the double bonds, gave a mixture of the unchanged diene (**2a**), the target compound, (*E*)-2,6-dimethyl-6-octen-2-ol (**4a**), 3,7-dimethyl-6-octen-3-ol (**5**), and 2,6-dimethyloctane-2,6-diol (**6**) in which the diols **4a** and **5** were present in an approximate ratio of 1:1.

Although the two trisubstituted double bonds in the diene **3a** are so similar that totally regioselective hydroxylation was not expected to be feasible, reverse addition, i.e., addition of the mercury(II) acetate to the diene according to Brown et al. (1972), gave the terpenols **4a** and **5** in a ratio of 91:9. A fraction containing the desired terpenol, **4a**, and the terpenol **5** in a ratio of 97:3 was isolated from the crude product by column chromatography. As far as could be ascertained, (*E*)-2,6-dimethyl-6-octen-2-ol (**4a**) is a new natural product for which the name (*E*)-subaeneol is proposed. In addition to butanoic acid, (*E*)-2,6-dimethyl-6-octen-2-ol, and skatole, which reproducibly elicited EAD responses in male and female antennae of *K. subaeneus*, some other constituents of the abdominal secretion in a few instances also gave EAD responses. Because of the nonreproducibility of the EAD results, the minor EAD-active constituents of the secretion could not be identified. However, the major volatile constituent of the secretion, eluting at 47.2 and 22.1 min in the gas chromatograms shown in Figures 1 and 2, respectively, also gave an EAD response in about 5–10% of the analyses.

This compound was identified as the chiral 2,6-dimethyl-5-heptenoic acid, which had previously been identified in *K. lamarcki* (Burger et al., 1983). (*R*)-(-)-2,6-Dimethyl-5-heptenoic acid (**9a**) was synthesized from commercially available (*R*)-(-)-3,7-dimethyl-1,6-octadiene [(*-*)- β -citronelene] (**7a**) by epoxidation of the trisubstituted double bond to give the unsaturated epoxide (**8a**), ozonolysis of the double bond in this compound, and simultaneous deprotection of the double bond (Knöll and Tamm, 1975) giving the unsaturated aldehyde (**9a**) and oxidation of the aldehyde to (*R*)-(-)-2,6-dimethyl-5-heptenoic acid (**10a**) according to Scheme 2.

Racemic 2,6-dimethyl-5-heptenoic acid could be resolved by gas chromatography on a capillary column coated with heptakis(2,3,6-tri-*O*-methyl)- β -cyclodextrin/OV-1701. It was found that the synthetic acid (**10a**) and the natural compound did not coelute from this chiral column. Therefore, the natural compound had to be (*S*)-2,6-dimethyl-5-heptenoic acid (**10b**). This enantiomer, which coeluted with the natural compound was synthesized from (*S*)-(+)-3,7-dimethyl-1,6-octadiene [(+)- β -citronellene] (**7b**) according to a sequence of steps similar to those used for the synthesis of the *R* enantiomer (**10a**), as shown in Scheme 2. Although the mass spectrum of the synthetic acid is identical to that of the natural compound and it coeluted with the natural compound from the chiral GC column, it did not elicit EAD responses in a large number of male and female antennae that were used in EAD/FID measurements.

This is not unexpected in view of the observation that the natural compound also did not reproducibly elicit EAD responses. From the research done with dung beetles over many years, it is clear that semiochemical communication in dung beetles is extraordinarily complex, and as far as the inconsistency of antennal responses in EAD analyses with the antennae of this and other *Kheper* species is concerned, there are various factors that could be taken into consideration. It is, for example, possible that there could be some natural variation in the population in terms of antennal responses. Because the research had to be done with captured insects, it was not possible to determine the age, physiological state, etc of the insects that were used in these experiments, factors that could have influenced the EAD results. The possibility that the response of an antenna to the volatile constituents of the secretion could depend on the concentration of the components flowing over the antenna was investigated, but this does not seem to explain the inconsistent results obtained with (*S*)-2,6-dimethyl-5-heptenoic acid (**10b**) and some of the other minor constituents of the secretion.

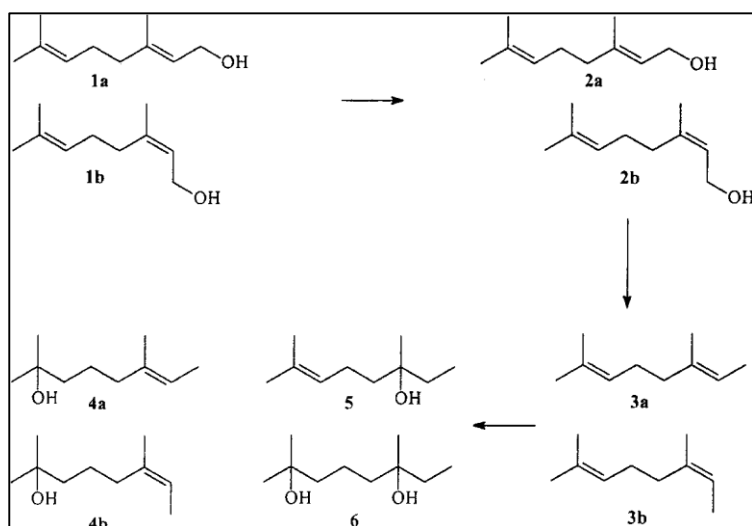
Another factor that has to be considered is that the best EAD results are mostly obtained when an EAD-active compound is eluted as a sharp peak from the GC column. Better results are often obtained when higher programming rates are used. Many of the volatile constituents of the secretion are carboxylic acids or other polar compounds that elute as more or less pretailing peaks from apolar columns. If an EAD active constituent is eluted as a pretailing peak, it is possible that the receptors on the antenna become saturated at a level below the detection threshold of the system. In the present investigation, a capillary column coated with OV-1701-OH was used because it is known that underivatized fatty acids are eluted with better peak shapes from this phase than from other apolar and even moderately polar stationary phases. A detailed investigation of the effect of the choice of stationary phase and programming rate on EAD/FID analyses with dung beetle antennae have, however, not yet been undertaken.

Using a modulating device, which delivers the effluent of the column as sharp pulses into the humidified airstream of the EAD setup, could possibly solve the problem of pretailing peaks in EAD analyses. Unfortunately, as in the research on *K. nigroaeneus* (Burger and Petersen, 2002), field tests with synthetic materials have so far not produced positive results. The problems associated with field tests of the synthetic attractant constituents of *Kheper* dung beetles were discussed in a previous communication (Burger and Petersen, 2002). The results obtained in interspecific EAD analyses revealed that considerable interspecific semiochemical communication could take place between dung beetles in the same area. This aspect will be dealt with in a forthcoming communication.

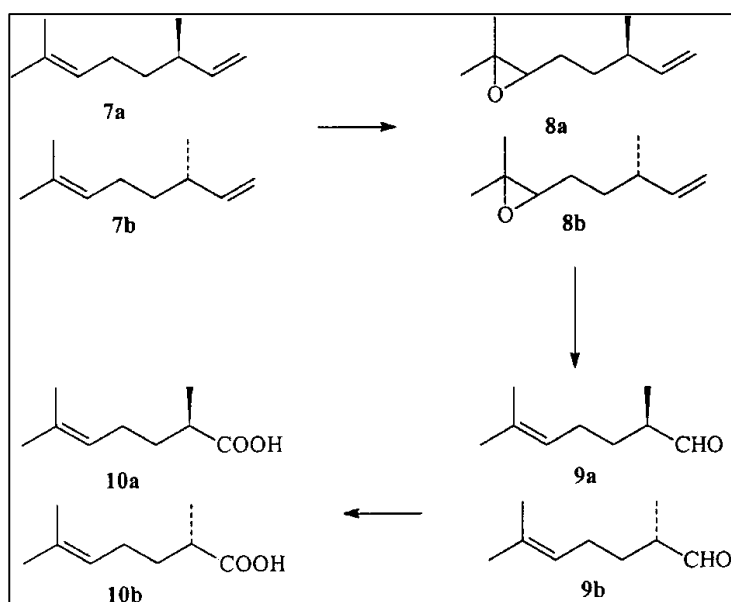
Acknowledgments

Support of this work by the University of Stellenbosch and the Foundation for Research Development, Pretoria, is gratefully acknowledged. The authors are indebted to the Natal Parks, Game, and Fish Preservation Board for permission to study dung beetles in Mkuzi Game Reserve.

Scheme



Scheme 1



Scheme 2

Figures

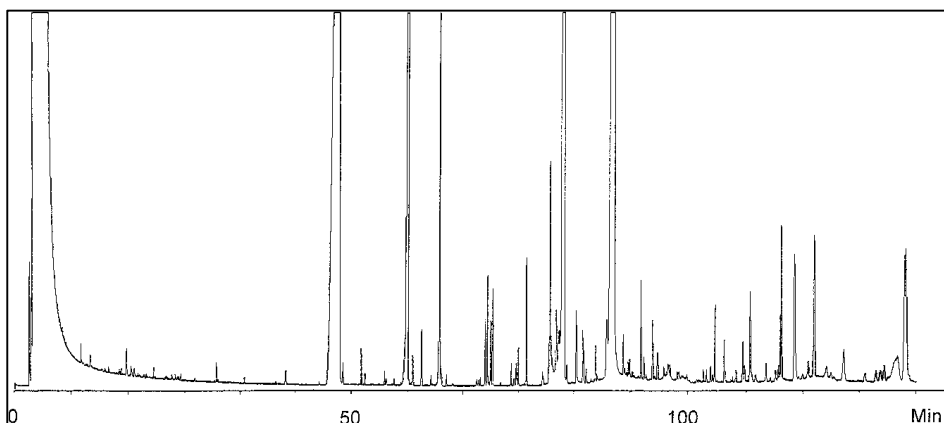


FIG. 1. Total ion chromatogram (GC-MS) of an extract of the male abdominal secretion of the dung beetle *Kheper subaeneus*. Gas chromatographic conditions are given in the text.

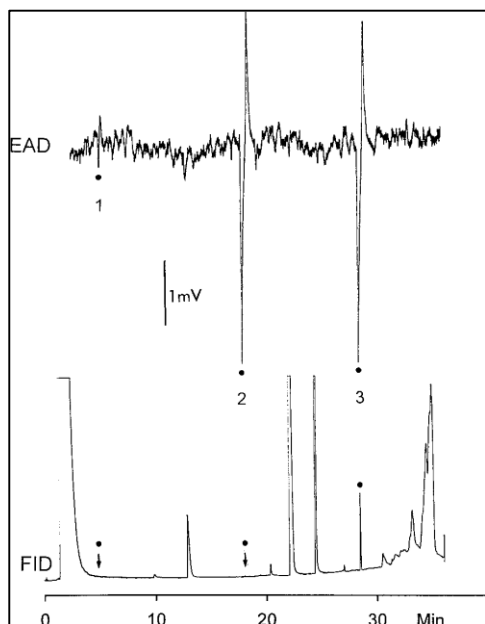


FIG. 2. First part of a gas chromatographic analysis of an extract of the male abdominal secretion of *Kheper subaeneus* with EAD and FID recording in parallel using a female antenna as sensing element. Chart speed was 0.5 cm/min. Gas chromatographic conditions are given in the text. The poor separation of later eluting peaks is due to overloading of the column. Assignment of EAD-active constituents: 1 = butanoic acid; 2 = (*E*)-2,6-dimethyl-6-octen-2-ol; 3 = skatole.

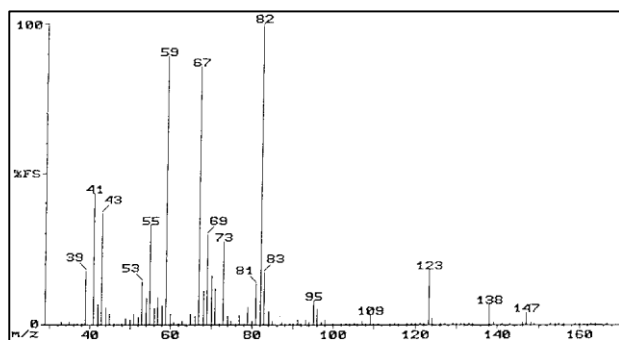


FIG. 3. Mass spectrum of the EAD-active constituent [(*E*)-2,6-dimethyl-6-octen-2-ol] of the male abdominal secretion of *Kheper subaeneus* eluting at 17.75 min in the gas chromatogram shown in Figure 2.

REFERENCES

1. BURGER, B. V. and PETERSEN, W. G. B. 1991. Semiochemicals of the Scarabaeinae III: Identification of the attractant for the dung beetle *Pachylomerus femoralis* in the fruit of the spineless monkey orange tree, *Strychnos madagascariensis*. *Z. Naturforsch.* 46c:1073–1079.
2. BURGER, B. V. and PETERSEN, W. G. B. 2002. Semiochemicals of the Scarabaeinae: VI. Identification of EAD-active constituents of abdominal secretion of male dung beetle, *Kheper nigroaeneus*. *J. Chem. Ecol.* 28:485–497.
3. BURGER, B. V., MUNRO, Z., ROTH, M., SPIES, H. S. C., TRUTER, V., TRIBE, G. D., and CREWE, R. M. 1983. Composition of the heterogeneous sex attracting secretion of the dung beetle, *Kheper lamarcki*. *Z. Naturforsch.* 38c:848–855.
4. BURGER, B. V., MUNRO, Z., and BRANDT, W. F. 1990. Pheromones of the Scarabaeinae II. Composition of the pheromone disseminating carrier material secreted by male dung beetles of the genus *Kheper*. *Z. Naturforsch.* 45c:863–872.
5. BURGER, B. V., PETERSEN, W. J. G., and TRIBE, G. D. 1995. Pheromones of the Scarabaeinae IV. Identification of an attractant for the dung beetle *Pachylomerus femoralis* in the abdominal secretion of the dung beetle *Kheper lamarcki*. *Z. Naturforsch.* 50c:675–680.
6. BROWN, H. C. and GEOGHEGAN, P. J., JR. 1967. The oxymercuration–demercuration of representative olefins. A convenient, mild procedure for the Markovnikov hydration of the carbon–carbon double bond. *J. Am. Chem. Soc.* 89:1522–1524.
7. BROWN, H. C., GEOGHEGAN, P. J., JR., LYNCH, G. J., and KUREK, J. T. 1972. Solvomercuration–demercuration. IV. The monohydration of representative dienes via oxymercuration–demercuration. *J. Org. Chem.* 37:1941–1947.
8. KNÖLL, W. and TAMM, C. 1975. Biosynthese der Verrucarine und Roridine. Teil 5. Synthese von zwei Diastereoisomerenpaaren des [1.8-14C]-⁶-Bisabolols und Versuche zu deren Einbau in Verrucarol. *Helv. Chim. Acta* 58:1062–1171.
9. VAN DER PERS, J. N. C. 1980. Olfactory Receptors in Small Ermine Moths (Lepidoptera : Yponomeutidae): Electrophysiology and Morphology. PhD thesis. University of Groningen, p. 39.